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14. ABSTRACT In order to study the role that estrogen might have in breast cancer DNA amplification, I started to build the cell lines that I will use as a model for the upcoming experiments. MCF7 Flp-In cell lines have been established through the selection of cells stably transfected to carry an FRT (Flippase Recombinase Target site) sequence, which subsequently will function as acceptor site for the integration of constructs with an engineered replication origin via the FLP/FRT method. The cell lines obtained were screened and characterized. Anticipating an experiment proposed for Year-Two, MCF7/cMyc 6xERE cells, which have an ectopic c-Myc replication origin engineered to contain estrogen receptor-alpha binding elements, were treated with 17-β estradiol for one month and subsequently exposed to a high dose of G418, whose resistance marker is encoded within the integrated pFRT_c-Myc 6xERE construct. Although only one preliminary experiment has been done, the drug selection allowed enrichment of the cells that have DNA amplification at the ectopic c-Myc origin after cells were exposed to estrogen.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusion.....	13
References.....	15
Appendices.....	16
Supporting Data.....	23

INTRODUCTION

DNA amplification is a hallmark of human cancer that can provide proliferative advantages to malignant cells, such as activation of oncogenes. Moreover, DNA amplification is often correlated with disease prognosis and progression. However, mechanisms that trigger DNA amplification are not yet fully understood. This research project aims to shed some light on the involvement of replication origins in DNA amplification. In particular, I want to explore whether the steroid hormone estrogen, when binding adjacent to a replication origin, can induce re-replication leading to DNA amplification in breast cancer cells. Towards this aim, cell lines that have an ectopic replication origin engineered with estrogen receptor-alpha binding elements will be used as a model system. Cells will be exposed to estrogen and DNA amplification at the engineered locus will be analyzed. Understanding the mechanism of DNA amplification is extremely important for the development of therapies aimed to counteract cancer cell proliferation and metastasis.

BODY

Methods

DNA Preparation. Small scale plasmid DNA was prepared using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. High scale plasmid DNA preparations were performed with PureLink HiPure Plasmid Filter Purification Kit (Invitrogen) following the manufacturer's instructions. Genomic DNA from human cell lines was isolated either by standard phenol/chloroform extraction or the Gentra Puregene Kit (Qiagen) following the manufacturer's instructions.

Establishment of Flp-In Cell Lines. Cells were transfected with pFRT/lacZeo plasmid (Invitrogen) linearized with XmnI restriction endonuclease (New England Biolabs) using FugeneHD Transfection Reagent (Promega) following the manufacturer's instruction. Briefly, a 3:1 FugeneHD (μL): plasmid DNA (μL) transfection mixture was incubated 10 min at room temperature and then added to log-growth phase cells about 80% confluent plated 24 h earlier. Transfection efficiency was assessed by detection of YFP expression in cells transfected at the same time with the pbabeYFP control plasmid. At 48 h after transfection, cells were selected with 200 $\mu\text{g/mL}$ Zeocin. Drug resistant clones were isolated and expanded.

Southern Blot. Southern blots were performed as described in Sambrook *et al.*, Molecular Cloning (1989). 10 μg genomic DNA isolated from MCF7 Flp-In cells were digested overnight at 37°C with 100 units of restriction enzyme (New England Biolabs) and run on a 1% agarose gel. The gel was sequentially soaked with gentle agitation in 0.25 HCl for 20 min, dH₂O for 2 min, and twice in Transfer buffer (0.4 M NaOH, 1 M NaCl) for 20 min. DNA was transferred onto a positively charged nylon membrane (Hybond XL, Amersham) by overnight capillary alkaline transfer using the Transfer buffer. Subsequently, the membrane was washed twice in 0.5 M Tris-HCl pH 7.2, 1M NaCl for 20 min, air dried, and baked at 80°C for 2 h. The membrane was pre-hybridized in Hybridization Buffer (0.5 M Na₂HPO₄ pH 7.2, 7% SDS, 1 mM EDTA) at 60°C for 4 h. The 1945 bp ³²P labeled probe (HindIII-SacI fragment of the pFRT/lacZeo vector) was synthesized using the NEBlot kit (New England Biolabs) following the manufacturer's instruction. Free ³²P was removed from the reaction by gel filtration (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare). The membrane was hybridized overnight at 60°C with Hybridization buffer supplemented with the radioactive probe (6×10^5 cpm/mL). The membrane was washed at room temperature 5 min with 2X SSC, 0.25% SDS, twice with 2X SSC, 0.1% SDS for 20 min, twice with 1X SSC, 0.1% SDS for 20 min. Finally, the membrane was exposed to X-Ray film at -80°C with intensifying screen for 1-4 days.

Quantitative PCR. 10 ng genomic DNA were assessed for each reaction using SYBER Green PCR Master Mix (Applied Biosystems). The amplification efficiency of each primer set was measured by testing serial dilutions of a reference sample. In each assay, samples were run in triplicate (expression level) or quadruplicate (DNA copy

number). Assays were performed with an Applied Biosystems 7300 Real Time PCR machine.

Mapping of the pFRT/lacZeo Genomic Integration Site. LAM-PCR was performed as described in Schmidt *et al.*, Nature Methods (2007). Inverse PCR was performed by digesting genomic DNA with a restriction endonuclease that cleaves the vector only once at 100-300 bp from the edge of the chromosomal insertion site. After phenol/chloroform extraction, the digested genomic fragments were self-ligated and used as a template to PCR amplify the genomic sequence adjacent to the integrated vector. PCR primers anneal within the 100-300 bp known vector sequence determined by the restriction enzyme used. Nested PCR was performed to increase amplicon abundance. The final PCR product was gel purified (QIAquick Gel extraction kit, Qiagen) and sent for sequencing. Both restriction enzyme and PCR primers were designed to be clone-specific. Results were confirmed by PCR.

Nascent Strand Isolation and Origin Activity Assessment. Total genomic DNA was isolated from $\sim 10^7$ exponentially growing cells using DNAzol (Invitrogen) following the manufacturer's instructions. Single-stranded DNA (ssDNA) was enriched by affinity chromatography through a benzoylated naphthoylated DEAE (BND)-cellulose column (Sigma). The resin was equilibrated with NET buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) and, after loading the genomic DNA, ssDNA was eluted with NET buffer supplemented with 1.8% caffeine. The enriched sample was 5'-end phosphorylated with T4 polynucleotide kinase. Subsequently, the contaminating ssDNA derived from broken DNA was degraded by overnight digestion with λ -exonuclease enzyme (New England Biolabs): only newly synthesized DNA molecules are protected from degradation by the presence of an RNA primer at their 5'-end. Contaminating Okazaki fragments (<500 bp) were subsequently removed by size fractionation on gels (1-2 kb). The assessment of origin activity at a given site was determined by quantitative PCR (qPCR). The abundance of sequences at the assayed replication origins relative to the DNA abundance at a site without replication origin activity was normalized to total genomic DNA.

Cell Cycle Analysis. Cells were plated in phenol red-free media supplemented with 5% charcoal dextran-treated fetal bovine serum. After 3 days, cells were treated with 10 nM 17- β -estradiol (E2) or vehicle (ethanol) for 24 hrs. Cells were harvested, ethanol fixed overnight at 4°C, and propidium iodide stained. Cell cycle analysis was performed with BD-CellQuest software.

Results

Task One – Establishment of Cell Lines

- **Sub-task 1a (months 1-3): Generation of MCF-7 and T47D Flp-In acceptor cell lines (i.e. stably harboring the FRT recombination site) by transfection and antibiotic clones selection.**

As described in the proposed project, MCF7 and T47D breast cancer cell lines have been transfected with the pFRT/lacZeo plasmid (Invitrogen) to establish Flp-In acceptor cell lines that stably carry a Flippase Recombinase Target (FRT) site. The FRT site will subsequently serve as the genomic integration site of the plasmid containing the c-Myc replication origin, either with or without the 6xERE (Estrogen Response Element) binding sequence. The FLP/FRT recombination system avoids position effects that could complicate the analysis of the results when comparing cell lines stably transfected with the two different constructs. Likely because of the low transfection efficiency of T47D cells (about 20%), I did not recover any T47D Flp-In clone out of three independent transfections. Nonetheless, from four independent transfections of MCF7 cells (transfection efficiency ca 60%), I isolated and expanded 20 drug-resistant clones.

- **Sub-task 1b (months 4-5): Assessment of the integration of a unique FRT site into the genome by Southern blots.**

After I assessed the presence of the FRT site in each clone by PCR, in a preliminary experiment I determined the number of integrated FRT sequences by quantitative PCR (qPCR). This first screening allowed the identification of 10 clones carrying a single integrated FRT site (Figure 1). qPCR results for some MCF7 Flp-In clones (namely MCF7/1B2, MCF7/1D; MCF7/2C; MCF7/3B2) were confirmed by Southern blots. Different restriction enzymes have been used to confirm the results (Figure 2).

Southern blot analysis took a longer time than expected since I had to troubleshoot detection sensitivity and poor efficiency of the capillary transfer. The detection sensitivity problem, which was due to a not fully-efficient labeling kit, was identified by dot blots, probing known DNA quantities spotted onto a nylon membrane, and doing a preliminary Southern blot in which I ran serial dilutions of a positive control (the pFRT/lacZeo vector; 1ng, 100 pg, 10 pg) and tested different hybridization conditions while synthesizing the radioactive probe with a more efficient kit. The poor efficiency of the capillary transfer reflected the thickness of the agarose gel: a too thick gel (i.e. >7 mm) substantially lowers the transfer efficiency.

- **Sub-task 1c (months 6-7): Mapping the FRT site in the engineered MCF-7 cell lines.**

Two different strategies that rely on the known sequence of the edges of the integrated plasmid were used to map the genomic integration site of the FRT sequence. The first method is the Linear Amplification-Mediated PCR (LAM PCR; Schmidt *et al.*, 2007), which is based on subsequent steps of **i)** isolation of a biotin-labeled linear PCR product containing the end sequence of the integrated vector and the adjacent unknown genomic sequence, **ii)** ligation of a linker, and **iii)** exponential PCR amplifications

(Figure 3A). The second method is called inverse PCR, which is based on PCR amplification of a self re-circularized restriction fragment containing one end of the integrated vector and the flanking genomic sequence at the integration site (Figure 3B). Surprisingly, initial sequencing data showed that the site used for plasmid linearization before cell transfection was still intact, revealing the need to identify the edges of the integrated pFRT/lacZeo plasmid to accomplish the mapping. To this end I designed thirteen overlapping PCRs that cover the entire 8106 bp vector sequence (Figure 4). This issue considerably stretched the expected time required to map the FRT integration site. PCR results summarized in Table 1 highlight the heterogeneity of the plasmid integration process: After re-circularization of the vector, the breakage point occurred at a different site in different clones and differs in the size of deleted vector sequence. Surprisingly, two clones (namely MCF7/2A1 and MCF7/3A) seem to have maintained an episomal circular plasmid. With these data, I designed clone-specific mapping protocols, using different restriction enzymes (LAM-PCR) and/or primer sets (LAM-PCR and inverse PCR) depending on the sequence of the integrated plasmid edges. For some clones (namely MCF7/1B2, MCF7/2A2, MCF7/3D1, MCF7/3D2) the mapping procedure revealed that PCR failures (Table 1) were due to the presence of vector rearrangements rather than breakage that allowed genomic integration of the vector. The mapping for these clones was therefore not possible since I could not have the required initial information about the sequence at the edges of the integrated plasmid. Table 2 presents the FRT mapping information I obtained.

- **Sub-task 1d (months 8-9): Array comparative genome hybridization (CGH) of MCF-7 host cell lines to verify the absence of pre-existing amplification at the FRT insertion site locus.**

Because of the delays experienced for completing Sub-task 1b (Southern blot assay of integrated FRT sequences) and Sub-task 1c (FRT genomic integration site mapping), I did not address this sub-task yet.

Task Two – Can Estrogen Drive DNA Amplification at the Ectopic c-Myc replication origin?

- **Sub-task 2a (months 10-11): Cell transfection and antibiotic selection of recombinant clones plus confirmation of integration of both the c-Myc and 6xERE/c-Myc origin constructs at the FRT site.**

Because of the previous technical experience I gained during a preliminary experiment I did in this first year (see below “sub-task 2b”), I decided to modify the originally proposed plasmids that will provide the ectopic c-Myc or c-Myc 6xERE replication origin for integration at the FRT site (Malott and Leffak, 1999; Figure 7). Details about the changes I decided to make to the constructs (Figure 5) are listed below:

- To provide a more advantageous and efficient drug selection, the blasticidin resistance marker replaces the neomycin marker. Cells with amplification at the ectopic DNA replication origin will be resistant to an increased drug concentration as compared to positive transformants lacking amplification because of the copy number gain of blasticidin cassette.

- To have a second selection marker that can allow a different selection strategy, TagGFP2 (Evrogen) will be inserted. If DNA amplification occurs at the ectopic locus, a gain in TagGFP copy number will provide an increase in fluorescence signal and cell candidates carrying DNA amplification can be separated by FACS (Fluorescence-Activated Cell Sorting). TagGFP will provide the opportunity for a faster and independent screening for amplification compared to drug selection.
- The 2A peptide is placed in between the two markers encoded in a bicistronic mRNA; subsequently the two distinct polypeptides will be synthesized through ribosome skipping. The 2A peptide ensures an equal expression level of the two markers (de Felipe *et al.*, 2006).
- The mouse PGK (phosphoglycerinaldehyde kinase) promoter when used in human cells has been reported to drive low transcription levels (Qin *et al.*, 2010). A low transcription level of the markers would be more advantageous to identify cells that harbor the amplified ectopic locus, as the amplified signal would be more readily discernible as greater than the unamplified signal (TagGFP), and the drug resistance of the recombinant cells before amplification could be more easily overcome to select for cells with amplification (blasticidin). Moreover, higher protein levels that derive from the amplification will not be selected negatively because of toxicity to the cells.

Cloning is under way to construct the pFRT_Myc_PGK_Bla2AGFP and pFRT_Myc6xERE_PGK_Bla2AGFP vectors. Because of this modification of the experimental procedure, aimed to greatly ameliorate the DNA amplification selection at the ectopic replication origin, sub-task 2a will be performed during the second year of my DOD-BCRP award, soon after the above described construct will be ready.

• **Sub-task 2b (month 12): Real time PCR of nascent strand abundance to confirm the replicator activity of the ectopic c-Myc origin and the engineered 6xERE/c-Myc origin, plus confirmation of ER-alpha binding at the 6xERE site by ChIP-quantitative PCR.**

In order to get preliminary data while preparing newly established breast cancer recombinant cell lines, I started some experiments with a monoclonal MCF7 Flp-In host cell line kindly provided by Dr. Yasuhiro Arakawa (Jikei University School of Medicine, Tokyo). This monoclonal cell line had already been tested by Southern blots and was determined to have a single copy of the pFRT/lacZeo vector (Arakawa *et al.*, 2009). To better characterize this MCF7 Flp-In cell line, I mapped the vector integration site. The mapping procedure was complicated by the fact that the FRT sequence donor plasmid used to establish the FRT host cell line (pFRT/lacZeo, Invitrogen) seems to have re-circularized before the integration took place, making random the breakage point in the vector. The concordant results from inverse PCR and LAM-PCR mapping techniques suggest that the FRT site in this cell line resides in the 5'-UTR of the estrogen receptor alpha gene (ESR1), where a previous genomic rearrangement is already present (Figure 6).

The monoclonal MCF7 Flp-In host cell line was used to integrate via FLP-mediated homologous recombination either the pFRT/c-Myc or the pFRT/c-Myc 6xERE construct (Figure 7). I engineered the 2.4 kb c-Myc replication origin core element (Mallott and Leffak, 1999) by placing six estrogen receptor binding sites in a region at

the 3' end of the c-Myc origin (nt 1932-2133, between the P0 and P1 promoter elements). Indeed, it was previously shown that this 200 bp sequence is dispensable for the c-Myc replicator activity (Liu *et al.*, 2003). The parent c-Myc origin plasmid was a gift from Michael Leffak (Wright State University, Dayton, OH). I established these recombinant MCF7/c-Myc and MCF7/c-Myc 6xERE cell lines.

As determined by qPCR, MCF7 are diploid for the ER-alpha coding gene (Figure 8), and to assay that recombinant cell lines had retained ER-alpha signaling, I measured the ER-alpha transcription level, demonstrating that ER-alpha is still expressed, even if at half of the level of the parental MCF7 Flp-In acceptor cell line (Figure 9). More important, these data demonstrate that the ER-alpha expression level is the same in the two recombinant cell lines (with and without 6xERE). Moreover, recombinant MCF7/c-Myc and MCF7/c-Myc 6xERE cells were tested to be still responsive to estrogen. FACS analysis showed that the construct integration site does not interfere with the estrogen signaling. Indeed the hormone treatment induces the cells to proliferate, while hormone starved control cells are arrested in G1-phase (Figure 10).

To assess the activity of the ectopic replication origins, I performed qPCR of nascent strands on genomic DNA of recombinant MCF7/c-Myc and MCF7/c-Myc 6xERE cells. Preliminary data to quantify replication origin activity using nascent strand abundance show that both the c-Myc origin with and without the flanking 6xERE function as a replication origin (Figure 11).

Anticipating an experiment proposed for the second year of my DOD-BCRP award (i.e. **Sub-task 2c (months 13-17): Culture the engineered test (c-Myc 6xERE) and control cells (c-Myc) with estrogen and select with high dose of antibiotic those cells that have amplified the engineered c-Myc locus**), I started to test the main hypothesis that estrogen can induce DNA amplification when acting near a DNA replication origin. MCF7/c-Myc 6xERE cells were treated for one month with 10 nM 17 β -estradiol (E2). Subsequently, cells were selected with 20 mM G418, a concentration that is 40-fold that necessary to kill MCF7 cells, and it is sufficient to kill recombinant MCF7/c-Myc 6xERE cells before E2 treatment (when only one copy of the marker gene is present). Indeed, if DNA amplification occurs at the ectopic locus, the G418 resistance marker would be amplified as well, allowing cells to be resistant to an increased drug concentration. The result of this preliminary experiment is really exciting: It shows that the drug selection allowed the identification of cells with an average of 13-fold relative amplification at the ectopic locus (Figure 12). Currently I am trying to repeat these results using both MCF7/c-Myc control cell line and MCF7/c-Myc 6xERE cell line.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of multiple MCF7 Flp-In cell lines through stable genomic integration of pFRT/lacZeo plasmid (Invitrogen);
- Evaluation of the copy number of integrated FRT sequences in MCF7 Flp-In cell lines by qPCR and further confirmation by Southern blots;
- Design of a new vector for establishment of recombinant MCF7/c-Myc_PGK-Bla2AGFP and MCF7/c-Myc 6xERE_PGK-Bla2AGFP cell lines;
- Preliminary experiments on MCF7/c-Myc and MCF7/c-Myc 6xERE recombinant cell lines established from a MCF7 Flp-In clone provided by Dr. Yasuhiro Arakawa (Jikei University School of Medicine, Tokyo):
 - Verified replicator activity of the genomic integrated c-Myc and c-Myc 6xERE replication origins by nascent strand abundance;
 - Verified the retained capability of the two recombinant cell lines to respond to 17- β estradiol signaling;
 - Completion of a preliminary experiment in which MCF7/c-Myc 6xERE cells have been treated with 10 nM E2 and subsequently selected with high G418 dose: Resistant cells have on average a 13-fold relative amplification at the ectopic c-Myc origin.

REPORTABLE OUTCOMES

- i. Manuscripts, abstracts, presentations: None
- ii. Licenses applied for and/or issued: None
- iii. Degrees obtained that are supported by this award: None
- iv. Development of cell lines, tissue or serum repositories: Breast cancer MCF7 Flp-In cell lines developed by stable integration of pFRT/lacZeo plasmid.
- v. Informatics such as databases and animal models: None
- vi. Funding applied for based on work supported by this award: None
- vii. Employment or research opportunities applied for and/or received based on experience/training supported by this award: None

CONCLUSION

During the first year of my DOD-BCRP award I established breast cancer MCF7 Flp-In host cell lines, and determined the copy number of integrated plasmid and its genomic integration site. The information I got identified three different MCF7 Flp-In clones that can be used for future experiments: In fact, these clones **i)** have a unique integrated FRT site that have been mapped, **ii)** referring to genome-wide data of ER-alpha binding sites in MCF7 cells (Carroll *et al.*, 2006), it seems that there are not endogenous ER-alpha binding sites in a 60 kb genomic window of the reference genome surrounding the FRT site (as reported by Hampton *et al.*, 2009, no genomic rearrangements have been found in MCF7 genome in these regions). This MCF7 Flp-In cell lines could be an extremely useful tool for the scientific community for obtaining single insertion of foreign DNA and also when an exact matched genetic background is needed as a control experiment. I also assessed the reliability of the ectopic c-Myc and c-Myc 6xERE origins, demonstrating the retention of replicator activity.

The promising preliminary experiments show the potential of this project. Indeed, DNA amplification at the ectopic c-Myc 6xERE origin has been detected after estrogen treatment and drug selection of a MCF7/c-Myc 6xERE cell line.

The relevance of this project is the elucidation of a possible mechanism for initiation of DNA amplification. This has future clinical importance due to the prevalence of DNA amplification in cancer (e.g. Myc oncogene is often amplified in multiple cancers and its copy number state correlates with disease prognosis).

Recommended Changes

1) As detailed in the Body Section, sub-task 1c I will modify the construct carrying the engineered c-Myc replication origin. The new pFRT/Myc_PGK_Bla2AGFP and pFRT/Myc 6xERE_PGK_Bla2AGFP constructs will provide more advantages compared to the originally proposed constructs: The PGK promoter would drive a basal low level transcription of the selection markers, avoiding toxicity due to increased endogenous protein level if amplification occurs at the ectopic locus. Moreover, the two selection markers (blasticidin and TagGFP) will provide a two-way selection of cells with amplification at the ectopic replication origin: 1) gain in blasticidin resistance gene copy number will increase the drug resistance threshold, allowing a drug-based enrichment of the cells that have undergone DNA amplification after estrogen treatment; 2) gain in TagGFP copy number would be traceable because of an increase in fluorescence signal; thus cells with amplification at the ectopic c-Myc origin could be identified and collected by FACS sorting.

2) Since getting T47D Flp-In host cells has been proved to be more difficult than expected, as a second model to integrate the engineered c-Myc DNA replication origin I propose to use an U2-OS cell line stably transfected with a tetracycline-inducible vector to express ER-alpha. I already received this cell line from Dr. Dale Leitman (Tee *et al.*, 2004). The availability of a second cell line model will further support and strengthen

experimental results about estrogen involvement in DNA amplification. Moreover, this model will provide great advantages to clearly test the role of estrogen in DNA amplification. Indeed, **i)** unlike MCF7, U2-OS cells do not depend on estrogen for cell growth, and cells will be exposed to estrogen only during the experiment period. If DNA amplification will be detected at the ectopic c-Myc origin, it will be more directly correlated to estrogen exposure. **ii)** Within this experimental setting, the same cell line, that is the U2-OS cell line stably transfected with the pFRT/c-Myc 6xERE_PGK_Bla2AGFP construct, will be used as both test and control. In fact, the control experiment will be performed in the absence of ER-alpha expression (no doxycyclin induction). The usage of the same cell line can prevent any possible difference between the test and the control cell line that could have been arisen during the clone selection process.

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APPENDICES

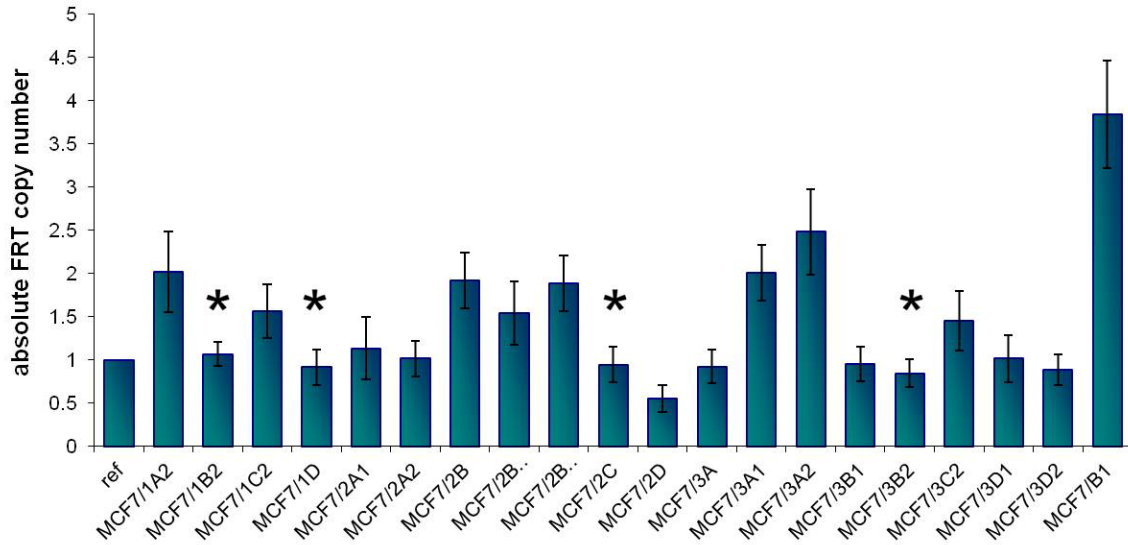


Figure 1. qPCR Estimation of Copy Number of Integrated FRT Sequence. The 20 MCF7 Flp-In clones derived after transfection with the pFRT/lacZeo plasmid were screened to identify those clones that have a single copy of the FRT sequence. The reference sample is the MCF7 Flp-In clone used by Arakawa *et al.* (2009), that was already screened by Southern blots. The error bar shows the standard deviation of 3 independent assays. Samples marked with an asterisk were used for Southern blots shown in Figure 2.

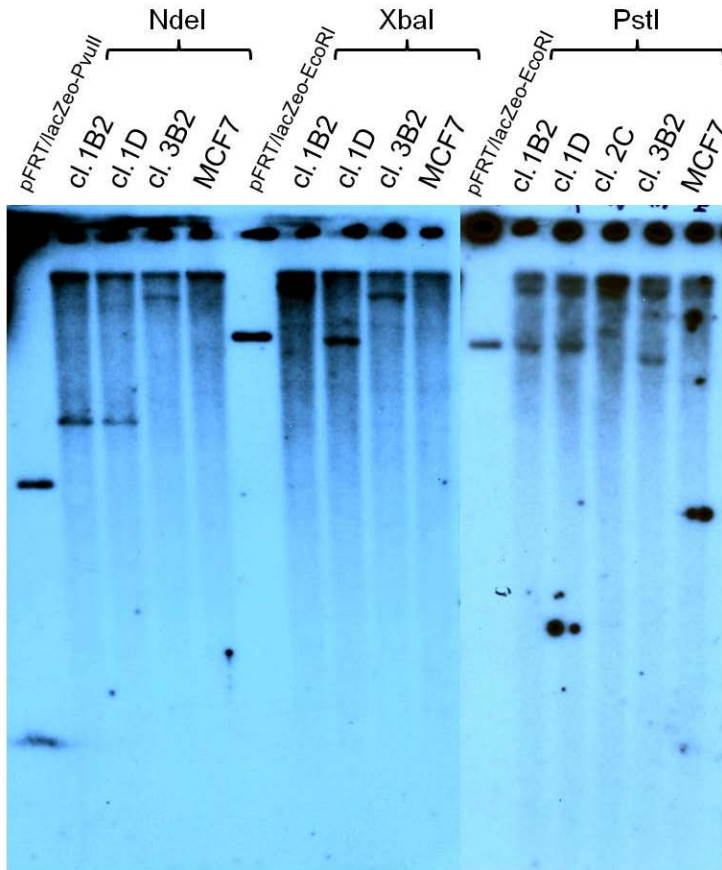


Figure 2. Southern Blots for Detection of Integrated pFRT/lacZeo. 10 μ g of genomic DNA of MCF7 Flp-In clones was digested with three different restriction enzymes (NdeI, XbaI, PstI). pFRT/lacZeo plasmid sequence was detected with a ~2 kb radioactive probe. As determined by qPCR, each of the clones analyzed has a single copy of the FRT carrying plasmid. Digested pFRT/lacZeo plasmid (10 pg) and MCF7 genomic DNA were used as positive and negative controls, respectively.

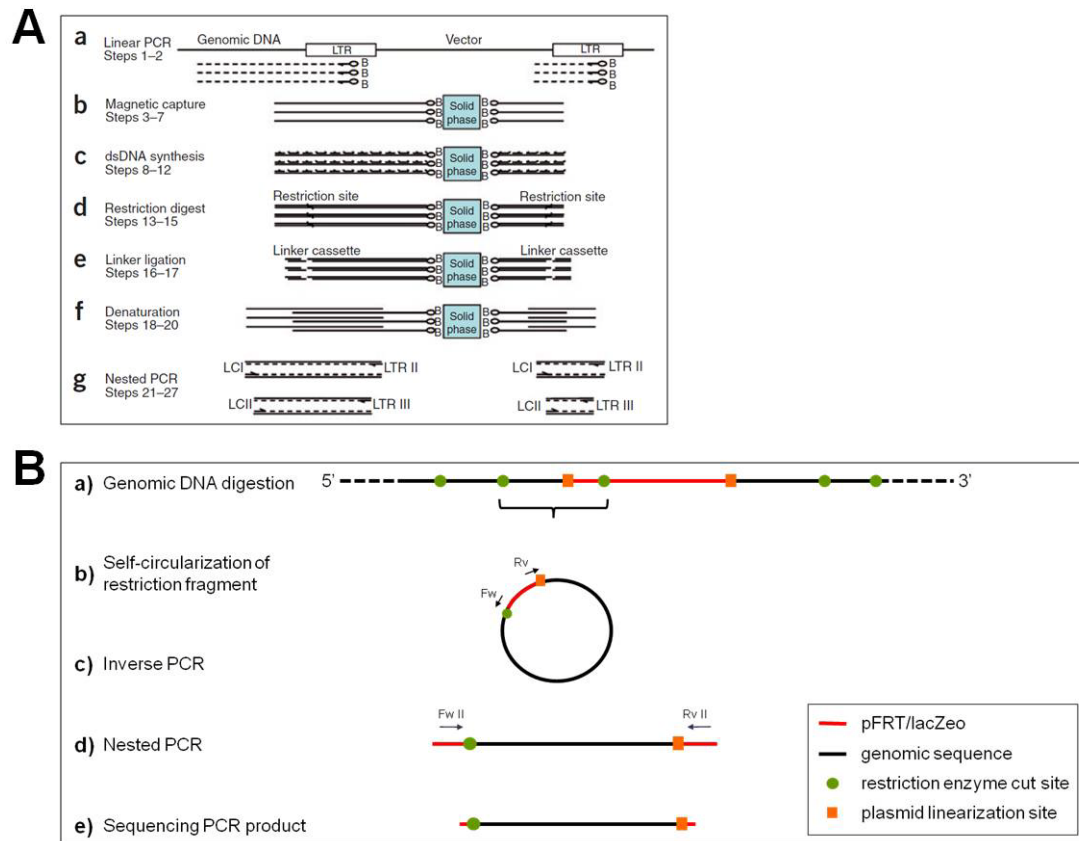


Figure 3. Schematic Representation of Strategies Used for Mapping Genomic FRT Integration Site. A. Linear Amplification-Mediated PCR (Schmidt M *et al.*, Nat Methods. 2007). **B.** Inverse PCR.

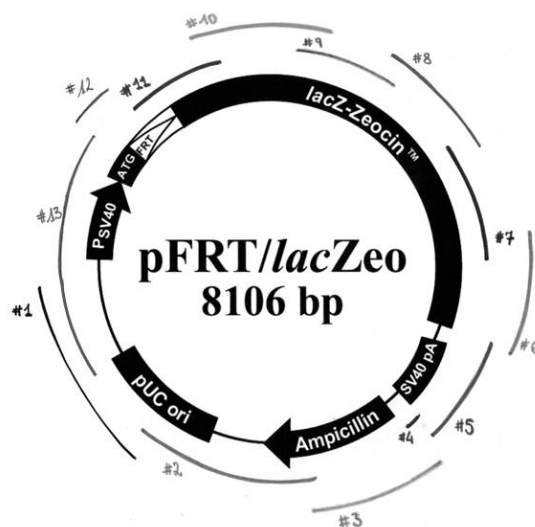


Figure 4. Scheme of pFRT/lacZeo Overlapping PCRs. 13 PCRs covering the entire pFRT/lacZeo sequence were designed to identify the boundaries of the integrated plasmid in MCF7 Flp-In clones.

		#1 (373 bp)	#2 (325 bp)	#3 (362 bp)	#4 (587 bp)	#5 (990 bp)	#6 (946 bp)	#7 (865 bp)	#8 (851 bp)	#9 (614 bp)	#10 (760 bp)	#11 (715 bp)	#12 (257 bp)	#13 (1581 bp)
	MCF7/1B2	ok	ok	no	no	ok	ok	ok	ok	ok	ok	ok	N/A	ok
*	MCF7/1D	ok	ok	no	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok
	MCF7/2A1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	N/A	ok
	MCF7/2A2	ok	ok	no	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok
*	MCF7/2C	no	no	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	no
	MCF7/3A	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok
	MCF7/3B1	ok	no	no	no	ok	ok	ok	ok	ok	ok	ok	N/A	ok
*	MCF7/3B2	no	no	no	ok	ok	ok	ok	ok	ok	ok	ok	ok	no
	MCF7/3D1	ok	ok	ok	no	ok	ok	ok	ok	ok	ok	ok	ok	ok
	MCF7/3D2	ok	ok	ok	no	ok	ok	ok	ok	ok	ok	ok	ok	ok

Table 1. Results of pFRT/lacZeo Overlapping PCRs. 13 overlapping PCRs were done on MCF7 Flp-In clones that have integrated a single copy of FRT sequence. PCR that failed (no) should identify the site where the plasmid opened to integrate into the genome. Asterisk shows clones used for further analysis in Table 2.

PCR-derived flanking genomic sequence						
Flp-In clone	chr	reference sequence	start	end	distance from FRT	graphical view
MCF7/1D	10p14	NC_000010.10	9038647	9038705	5799 bp	
MCF7/2C	3q26.1	NC_000003.11	160936128	160935500	783 bp	
MCF7/3B2	10p14	NC_000010.10	9345977	9346339	409 bp	

Table 2. Genomic Insertion Site of Three MCF7 Flp-In Clones . Inverse PCR and LAM-PCR were used to identify the pFRT/lacZeo genomic integration site.

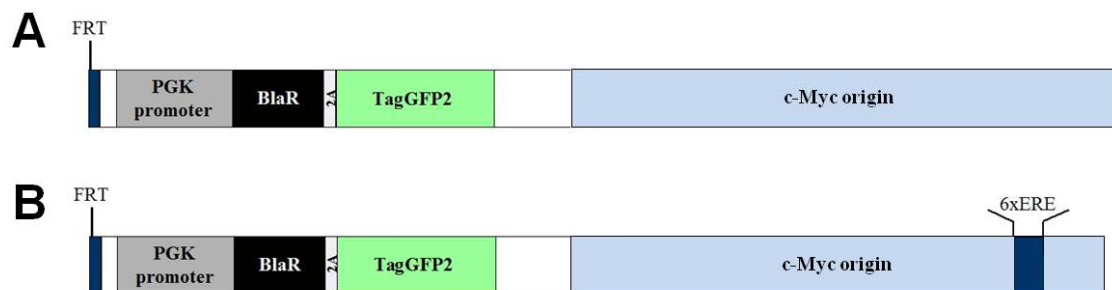


Figure 5. Scheme of A) pFRT/c-Myc_PGK_Bla2AGFP and B) pFRT/c-Myc 6xERE_PGK_Bla2AGFP Constructs. The modified constructs to be integrated in the newly established MCF7 Flp-In cells will have i) a mouse PGK promoter for a low level transcription, ii) a blastidicin resistance marker for a drug-based selection of cells after 17 β -estradiol treatment, iii) TagGFP2 (Evrogen) for fluorescence-based selection, iv) the 2A peptide that ensures an equal transcription level of the two markers, which are expressed as a bicistronic transcript.

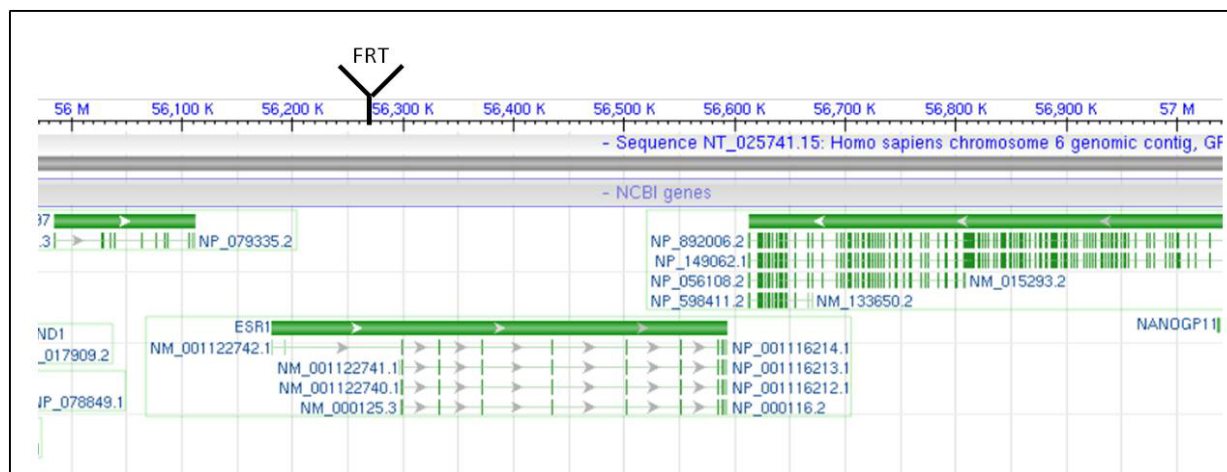


Figure 6. Graphical View of the FRT Sequence Integration Site in the Monoclonal MCF7 Flp-In Cell Line. Sequencing data demonstrate that in this MCF7 Flp-In clone (Arakawa *et al.*, 2009) the FRT (Flippase Recombinase Target site) donor plasmid integrated within the 5' UTR sequence of the ESR1 gene, coding for ER-alpha. Moreover, the sequencing data revealed the presence of an intra-chromosomal rearrangement 30 bp downstream of the integration site (Image from NCBI-Sequence Viewer).

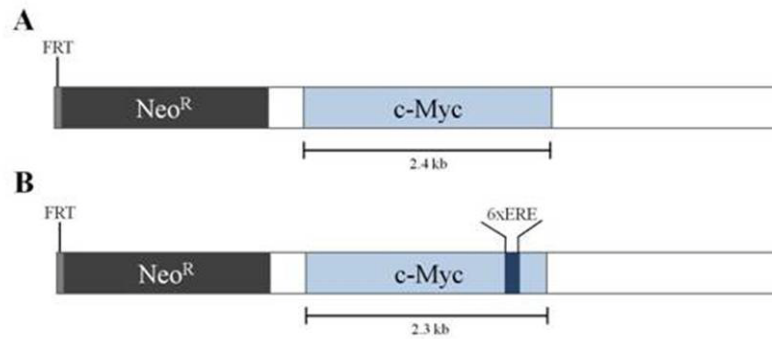


Figure 7. Scheme of A) pFRT/c-Myc and B) pFRT/c-Myc 6xERE Constructs. The FRT sequence, present in both the constructs and integrated into the MCF7 Flp-In host cell line, mediates the homologous recombination that is catalyzed by the flippase recombinase. The Flp/FRT recombination allows the integration of the two constructs at the same genomic position, avoiding positional effects to complicate interpretation of results. The six estrogen response elements (6xERE) replace a 200 bp fragment already shown to be dispensable for the c-Myc origin replicator activity.

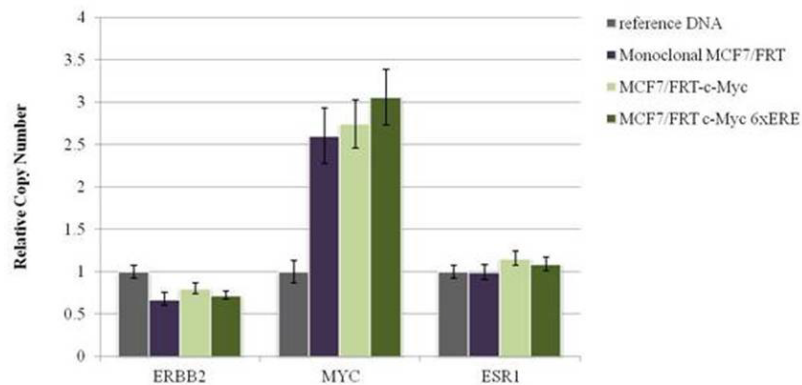


Figure 8. qPCR Measurement of ESR1 Copy Number in MCF7 cells. MCF7 Flp-In cells, as well as the recombinant cell lines MCF7/FRT c-Myc and MCF7/FRT c-Myc, have two copies of the ER-alpha coding gene. TBP (TATA box Binding Protein) gene was used as two-copies reference gene. Reference DNA is a commercial human genomic DNA (Promega). MYC and ERBB2 copy number state was evaluated as a control.

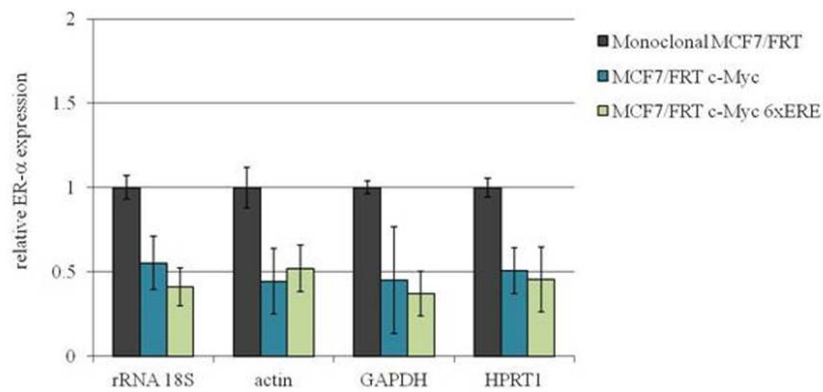


Figure 9. ER- α Expression in MCF7/FRT c-Myc and MCF7/FRT c-Myc 6xERE. mRNA level of ER- α was measured by qPCR using four different housekeeping genes as reference (rRNA18S, actin, GAPDH, HPRT1). Even if ER- α expression level in MCF7/FRT c-Myc and MCF7/FRT c-Myc 6xERE cell lines is reduced compared to the parental MCF7 Flp-In cells, ER- α is expressed at the same level in the two recombinant cell lines.

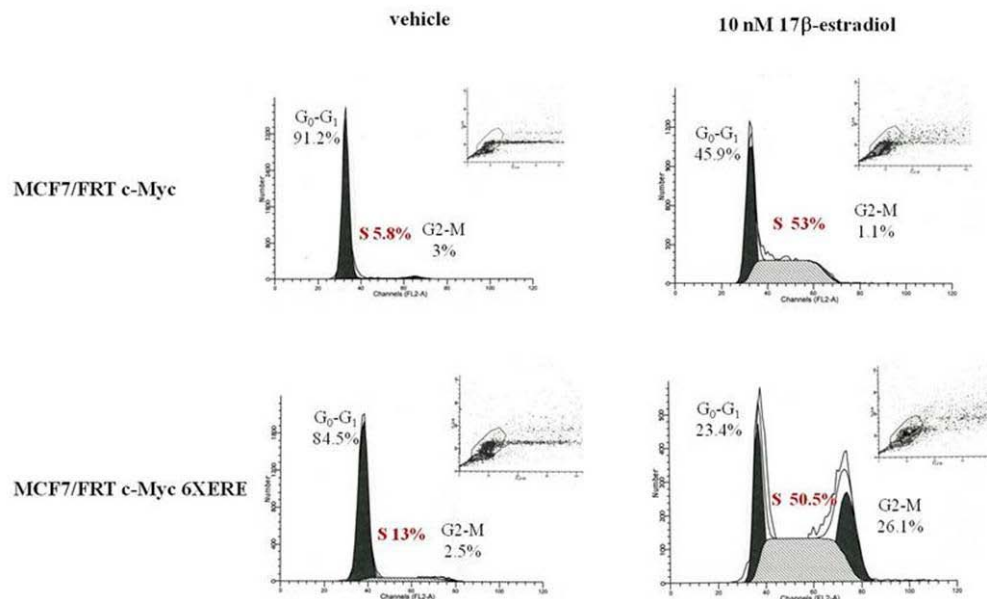


Figure 10. Response to 17 β -estradiol Treatment. MCF7/FRT c-Myc and MCF7/FRT c-Myc 6xERE respond to E₂ treatment, with about 50% of the cells in S phase after 24 hours treatment, while control cells remain growth arrested in G₁ phase.

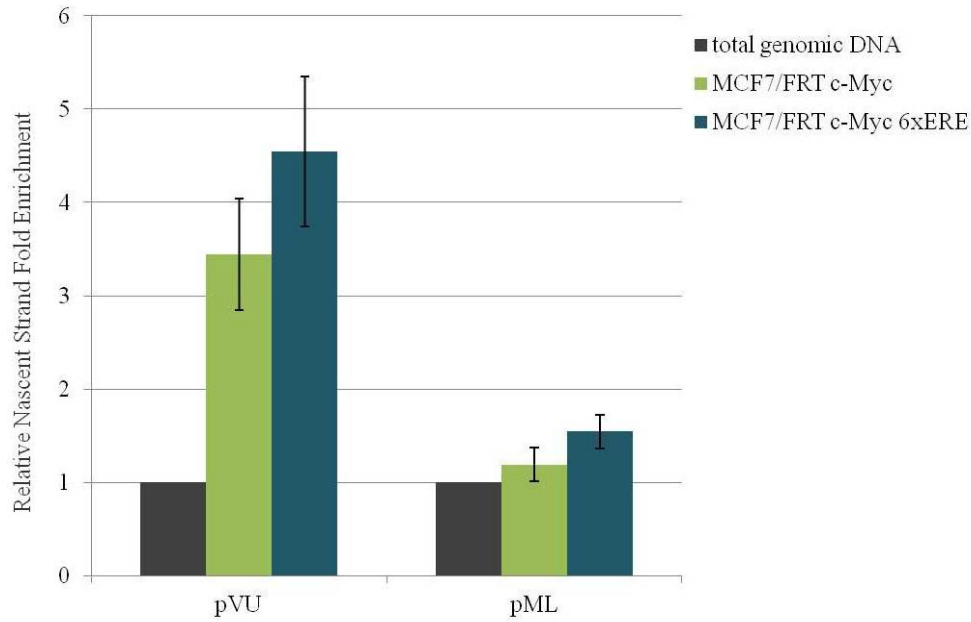


Figure 11. Evaluation of Replicator Activity at the Ectopic c-Myc Origin. The enrichment of two 100 bp vector-specific sequences located just upstream (pUV) or downstream (pML) of the ectopic c-Myc replication origin was measured by qPCR. Both c-Myc and c-Myc 6xERE origins retain their replicator activity at the MCF7 Flp-In integration site, with a 3.5 – 4.5 fold enrichment of the pVU sequence, respectively. Considering that the ectopic c-Myc origin is 2.4 kb long, the absence of enrichment at the pML sequence is probably due to the 1 to 2 kb size selection of the newly synthesized DNA strands.

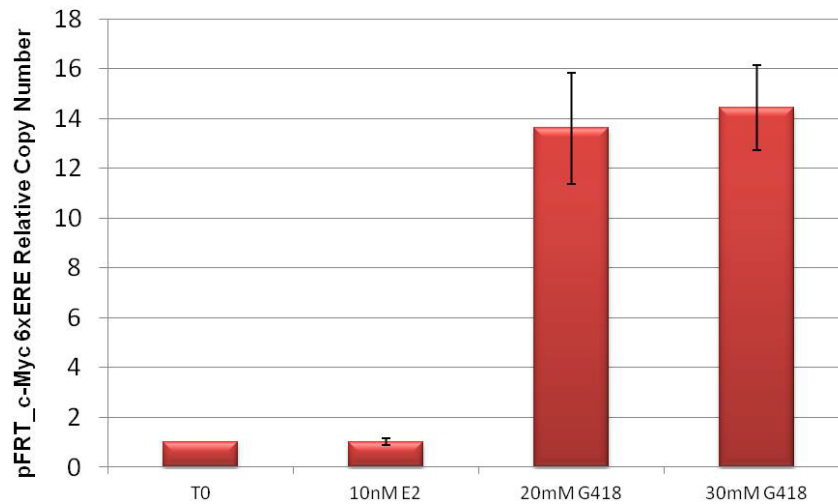


Figure 12. Evaluation of DNA Amplification at the Ectopic c-Myc Origin. MCF7/FRT c-Myc 6xERE were treated for 1 month with 10 nM E2 and then treated with 20 mM G418 and 30 mM G418. Genomic DNA was isolated from cells collected at time zero (T0), after the steroid hormone treatment (10 nM E2), and from cells survived to the high dose G418 selection (20 mM G418 and 30 mM G418). The vector specific sequence was normalized to TBP (TATA Binding Protein).

SUPPORTING DATA

None